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Richmond, Virginia

To: Mr. A. C. Lilly

Date: July 27, 1988

From: C. Ellis and M. Penn

Subject: Operational Plans for the Lowered Biological Activity Program Updated from Second Quarter Planning Meeting

OBJECTIVE: To decrease the biological activity of cigarette smoke condensate (CSC) by 90% as determined by in vitro assays by a target date of 1992.

BACKGROUND AND STATUS: Based on information in the literature, biological activity can be divided into several stages: initiation, promotion, and conversion. In addition, various biochemical factors may modulate the activity of a substance. The strategies used in the Lowered Biological Activity Program have been to: obtain information on which stage(s) may be important to the activity of CSC; develop in vitro bioassays to quantitatively measure CSC activity; evaluate cigarette models in the assays to obtain background information; develop and test models which are reduced in activity; and optimize the subjectives of a low activity model.

After many years of investigating assays for their ability to detect initiation activity, the Salmonella/microsome (S/M) assay was determined to provide the best information for our purposes. In addition, the S/M assay is the most widely used and accepted in vitro bioassay in the scientific community. The S/M assay is currently being used in the Crossed Solubles/Base Web Study to develop model cigarettes with reduced activity. Feedstock, base web, and all possible RL combinations of the solubles and base webs were made from burley, bright and oriental tobacco. Extensive analytical data have been obtained in these models and, based on this information, modifications have been made to the solubles by a variety of methods. The effect of these modifications on activity is evaluated by spraying the solubles onto the base web, fabricating cigarettes, and testing the CSC in the S/M assay. Significant reductions in activity have been obtained with various modifications to the CEL and an active research program is planned in this area.

Based on the lower S/M activity models that have been developed, work will need to be done to improve the subjectives of these cigarettes. Studies involving the optimization of low activity models have concentrated on the reduction of carbonyls in smoke. This work has been successful and future studies will focus on the reduction of NO_x in smoke.

The Glutathione Depletion Assay (GDA) is a biochemical endpoint that is utilized to evaluate the effect of CSC on glutathione depletion. Glutathione (GSH) is a low molecular weight thiol that is important to normal cellular functions and biological defense mechanisms. Depletion of GSH has been demonstrated to produce an increase in the activity of a positive control

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compound in the S/M assay. Plans in this area include the evaluation of a variety of models and the isolation and identification of substances in CSC that are responsible for GSH depletion.

The development of a promotion assay for CSC continues to be a key goal of the Lowered Biological Activity Program. The central focus of the potential promotion assays under development is the enzyme protein kinase C (PKC) which can modulate cell physiology and biochemistry via the phosphorylation of cellular proteins. The two binding assays that are currently being investigated are the epidermal growth factor (EGF) binding assay and the phorbol 12, 13 - dibutyrate (PDBu) binding assay. The results obtained with the EGF assay indicate that CSC obtained from several cigarette models can inhibit EGF binding to its receptor. Investigations regarding the assay's ability to differentiate among selected CSCs are in progress. Optimization of assay parameters and kinetic studies for the PDBu binding assay have been completed. Experiments have been conducted with CSC and the results indicate that 2R1 CSC stimulates the binding of PDBu. Two additional assays are being investigated for their ability to measure the effect of CSC on protein kinase C in cell fractions and intact cells.

Because some aspects of biological activity are poorly understood and are the subject of intense investigation in the scientific community, additional leads may become available to us at any time. New information could drastically affect our plan. We intend to monitor the literature very closely and make modifications to the program when the evidence warrants a change.

STRATEGIES:

1. INFORMATION SURVEY: maintain an awareness of scientific advances related to the understanding of biological activity and the implication of a role for CSC.

2. BIOASSAY DEVELOPMENT: develop suitable in vitro bioassays which can clearly differentiate among a series of model cigarettes.

3. MODEL EVALUATION: determine the activity of smoke condensate from various types of cigarette models in order to obtain background information from which to design modifications.

4. MODEL DEVELOPMENT: perform modifications to various cigarette parameters to reduce activity and formulate new models.

5. MODEL OPTIMIZATION: improve the subjectives of a low activity model.

TACTICS AND TIMETABLE:

OPERATIONAL PLANS FOR 1988

I. BIOASSAY DEVELOPMENT

A. Epidermal Growth Factor Binding Assay

Third Quarter 1988
1. Determine effect of removing catechol from CSC. (August 1)
2. Compare EGF from Amersham and Collaborative. (August 1)
3. Complete time course and fraction study. (August 1)

Fourth Quarter 1988
1. Test catechol depleted CSC and fractions. (October 1)
2. Using the EGF assay, investigate the activity of other classes of promoters. (October 1)
3. Begin to examine the pathway through which CSC inhibits EGF binding to aid in the development of a biochemical assay. (October 1)

Decision Point on EGF Assay: December 1988

B. Inhibition of Phorbol 12,13 -Dibutyrate (PDBu) Binding

Third Quarter 1988
1. Examine the effect of CSC on the affinity and number of PKC receptors. (July 31)
2. Examine effect of other CSCs. (August 31)
3. Examine effect of catechol on affinity and number of receptors. (September 30)

Fourth Quarter 1988
1. Examine possible synergistic effects. (October 31)

Decision Point on PDBu Assay: December 1988

C. Protein Kinase C (PKC) Activity Assay

Decision Point Reached:
Assay discontinued due to variability and high solvent background.

D. Protein Kinase C Quantitation Assay
Utilize an antibody to quantitate enzyme levels/location.

Third Quarter 1988
1. Complete development/optimization of techniques. (July 31)
2. Utilize antibody to quantitate enzyme levels and location. (August 31)
3. Examine effect of CSC. (September 31)

Decision Point on the PKC Quantitation Assay: December 1988

E. Protein Kinase C Activity in Intact Cells

Third Quarter 1988
1. Complete a preliminary investigation of the effects of CSC. (July 31)
2. Investigate the utilization of an antibody against phosphorylated tyrosine as another means of quantitating PKC activity. (September 30)

Fourth Quarter 1988

1. Determine effect of a promoter and CSC on phosphorylation of existing and newly synthesized proteins. (November 30)
2. Perform complete study on CSC and fractions. (Begin December 1)

First Quarter 1989

1. Perform additional experiments as needed on CSCs or fractions. (1989)

Decision Point on the PKC Activity Assay in Intact Cells: Second Quarter 1989

II. MODEL EVALUATION

A. Salmonella/microsome (S/M) Assay

Third Quarter 1988

1. Investigate effect of sugar-chlorogenic acid or sugar-caffeic acid complexes on 2R1 CSC activity. (September 30)
2. Evaluate other models and submitted samples. (ongoing)

B. Glutathione Depletion Assay (GDA)

Third Quarter 1988

1. Determine of effects of GSH depletion by CSC on activity measured in the S/M assay. (Complete September 30)

Fourth Quarter 1988

1. Evaluate the activity of HCN in the GDA. (November 15)
2. Begin isolation and identification of GSH-CSC adducts. (November 15)

III. MODEL DEVELOPMENT

A. Crossed Solubles/ Base Web Study

Third Quarter 1988

1. Complete investigation of the effect of monovalent and divalent cations. (July 15)
2. Complete ultrafiltration study. (July 31)
3. Determine the effect of CEL insolubles on activity. (August 31)
4. Complete ion exchange study:
   a. Generate additional material for extensive analysis. (August 31)
   b. Investigate effect of anion exchange resin. (September 30)
   c. Investigate effect of other ion exchange materials (Sepracor, other resins). (on-going)

Fourth Quarter 1988

1. Determine the effect of electrodialysis on the activity of tobacco solubles. (October 31)
2. Begin investigation of effects of nitrogenous compounds on

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activity (amino acids, proteins, nitrate). (Begin September)

3. Begin to investigate the effects of combining various modifications. (December 1)

IV. MODEL OPTIMIZATION
DECREASE LEVELS OF NO_X IN SMOKE

Third Quarter 1988

1. Optimize quantitative method for determining NO levels in smoke. (July 15)
2. Investigate effect of spraying a diene generator (vitamin A derivative, squalene, neophytadiene) onto filler on smoke NO levels. (August 31)
3. Investigate effect of oxidation of NO to NO_2 and then removal of NO_2 with phenoxide ions via charge transfer. (September 30)

EXTENDED PLANS:

BIOASSAY DEVELOPMENT:

1989: Begin to investigate other biochemical endpoints and techniques (mouse epidermal cell cultures, free radical mechanisms, intracellular calcium). Investigate other endpoints as evidence warrants. Begin to develop flow cytometry techniques.

1990: Utilize Flow Cytometry to increase our sensitivity, efficiency, and capabilities with regard to in vitro bioassays.

1991: Develop an assay for promotion activity. Investigate the activity of CSC in assays measuring activity in other stages of Biological Activity.

MODEL EVALUATION:

1989-1992: Continue to evaluate models as needed.

MODEL DEVELOPMENT:

1989-1990: Combine all effective modifications to obtain a low activity model for the S/M assay. Begin extensive testing. Begin flavor work.

1991: Begin the development of low promotion activity models.

MODEL OPTIMIZATION:

1989-1990: Optimize the subjectives of a low S/M activity model.

1991: Begin work on optimizing the subjectives of a low promotion activity model.

RESOURCE ALLOCATIONS FOR 1988:

Personnel Allocations:

INFORMATION SURVEY: Responsibility of all Professionals

BIOASSAY DEVELOPMENT:

M. Penn, Research Scientist, Project Leader(40%)
B. Davies, Research Scientist (60%)

G. Nixon, Scientist (100%)
G. Patskan, Scientist (100%)
D. Stagg, T4 (on special assignment)
T. Burruss, T3 (100%)
B. Vaughan, T3 (80%)

MODEL EVALUATION:

S/M Assay:
L. Thompson, Scientist (100%)
S. Coleman, T3 (60%)
C. Deubler, Temp (50%)
B. Vaughan, T3 (5%)
M. Penn, Research Scientist, Project Leader (10%)

GDA:
B. McCoy, Research Scientist (100%)
M. Penn, Research Scientist, Project Leader (10%)
B. Vaughan, T3 (15%)

Smoke and Sample Preparation:
R. Hellams, Research Scientist (40%)
N. McGee, T2 (45%),
R. Kinser, Research Scientist, Project Leader (5%)

MODEL DEVELOPMENT:

Crossed Solubles/Base Web Study:
D. Williams, Research Scientist (25%)
D. Magin, Research Scientist (80%)
S. Coleman, T3 (25%)

Smoke and Sample Preparation:
R. Hellams, Research Scientist (40%)
N. McGee, T2 (45%)
R. Kinser, Research Scientist, Project Leader (5%)

MODEL OPTIMIZATION:

NO_x:
R. Levins, Associate Senior Scientist (65%)
R. Kinser, Research Scientist, Project Leader (10%)

Number of People:

There are needs in the following areas:

1. A replacement for D. Stagg (T4) who is on special assignment.
2. A Chemist (replacement for David Williams/Don Magin who was on loan to us from Chemical Research/ARD) with some biology experience (Scientist or above) who can perform the following: crossed soluble/base web modifications and separations; coordinate model optimization (flavors), assist with the isolation and identification of GSH adducts, provide general assistance and chemical expertise to the Lowered Biological Activity Program.
3. A Chemist (Associate B level) who can assist in model development: the preparation of model cigarettes, spraying filler with modified CEL or other substances of interest, preparing cigarettes, preparing LTF formulations, and performing chemical analyses on filler and smoke. The individual would

work with R. Hellams and assist in research programs on the effect of filtration and smoking parameters on biological activity.

Anticipated need in 1989 is:

1. An individual with experience in flow cytometry. This relatively new technique could greatly enhance the sensitivity, efficiency, and capability of our bioassays and would impact Bioassay Development and other related areas of research. It is estimated that we could utilize these skills in 1989. A thorough review of the capabilities of this technique will be made in 1988 and an extensive justification will be written.

Skills of People:

See above.

Special Equipment and Facilities:

A Flow Cytometer (about $500K) could be utilized in 1989. This piece of equipment would have a significant impact on the sensitivity, efficiency, and capabilities of much of the biology/biochemistry related work. A thorough justification will be developed in 1988.

Outside Expertise:

Occasional antibody work may need to be performed.

Impact on Other Areas of PM:

1988:
Analytical Research: 200 man hours/year
Cigarette Testing: 30 man hours/year
Pilot Plant: 2 weeks every two years
Semiworks: approximately 1/2 day/year
Library
CAD

Extended:
Chemical Research: flavor development, beginning in 1989
NMR/MS(probably LC or FAB): for the isolation and identification of glutathione-CSC adducts, beginning in 1989
Flavor Development: assistance beginning in 1990

cc: J. L. Charles
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